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**Final report by Fritz Vollrath, Universities of Oxford UK and Aarhus DK**

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**Stress/strain characteristics and biochemical correlates of spider silks**

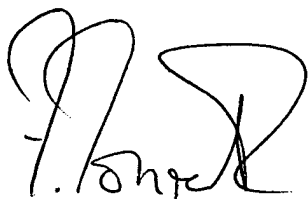
**Abstract of proposal:** Spider orb webs are high energy absorbing nets, and their ability to function well depends not only on the structural properties of web design but also on the material properties of the silk. A study using state of the art computer analysis and modelling will show how the spider's web absorbs the high kinetic energy imparted by wind load and prey impact. One important constraint for the spider is its building material, the silk, a biopolymer with very interesting mechanical properties. We have developed a special instrument to measure the stress/strain characteristics of this very fine material in great detail. We propose to employ our rapid response stress/strain gauge to measure the mechanical response of spider silks produced by a wide range of spiders both of different populations and different species. Together with collaborators at the Natick US Army Laboratories we also propose to do aminoacid analysis on silk samples measured and collected from the same spiders.

**Background to reports 1 and 2:** Dr Kaplan left Natick during the early stages of the project and took aspects of the proposed project with him to do elsewhere. Also, in the meantime (not least because of work done in my laboratory during this time under this contract) new results showed that intra-individual variability in silk properties can be as pronounced - or even more defined- than inter-specific variability. Thus together with Drs. Mello, Acardiacono and Fossey as representatives of the collaborating Natick laboratory) I decided to change focus and study instead of variability between

species the variability within one species. After all, first data collected during this study and elsewhere had shown that environmental conditions have huge effects on silk material properties (shown by us) as well as measurable effects on silk aminoacid compositions (C Craig pers. comm). Thus after initial samples of silks from a range of spiders (see reports 1 and 2) we began focused on silk from spiders kept under highly controlled conditions.

**Report 3 and 4:** In the second half of the contract my assistant Bo Madsen and I measured the mechanical properties of *Nephila* spiders that were starved or fed very specific diets. The results were striking, there was a significant effect (see attached paper presented at the 2nd International Charlotteville silk conference). We send samples of the silk measured to Natick for molecular analysis. Discussions with Drs Fossey and Acadiacono left us with the impression that their analysis could not resolve these differences nor could their results lead to any clear interpretation. We therefore decided to continue our mechanical studies of the effect of environmental conditions on silk feedstock production and fibre spinning with the hope of future improvements in the chemical analytical side. Obviously, the results of our highly detailed mechanical studies indicate clearly that there are chemical differences in silks produced by the same spider on successive days, depending on the environment. Indeed, very recent results (done under another study) vindicate this belief as they show that molecular composition and orientation is affected by the environmental conditions during spinning (unpublished observations).

**Conclusions:** This study was highly topical and produced good results. Due to the departure of our initial principal collaborator at Natick we never got the second part of the project to produce the firm intermeshed results that could have lead to a follow-up project fully funded by Natick. Still, we have made a few very important contributions to the understanding of spider silk properties. Especially, we can conclude that spinning conditions are very probably as important than the exact chemical composition of the feedstock. This has obvious commercial implications, namely that studying the spinning conditions in the spider is probably more rewarding than patenting yet another spider silk protein. Thus our study was a success because we initially set out to go the route of doing exactly that: identifying more spider silk proteins.

A handwritten signature in black ink, appearing to read 'F. Vollrath', with a stylized, looped design.

Fritz Vollrath

encl. Appendix

**Variability in the mechanical properties of spider silks on three levels: interspecific, intraspecific and intraindividual.**

Talk presented at 2nd International Silk conference in Charlottesville organised by Dr W. Adams et al. and funded by the US Army and Airforce.

**Abstract:** We examined the stress-strain characteristics of dragline silks from a range of spiders and found large inter- and intraspecific differences. Moreover, we observed daily variability in silk from individuals of *Nephila edulis* and we can show that spider condition (starvation) affected silk properties, in particular that it tended to decrease breaking elongation. Reeling speed significantly affected silk properties in both *Nephila edulis* and *Araneus diadematus*, such that with increasing speed (i) breaking elongation decreased, (ii) breaking stress increased and (iii) Young's modulus increased. Analysis of our data from the different reeling speeds indicates that *Nephila* and *Araneus* dragline silks differ in basic properties.

### **1. Introduction**

Spider silks are materials with a long history of evolution consisting of innumerable cycles of modification and selection. We must assume not only that the silks have been optimised for the main conditions which they might encounter but also that they have been customised to a wide range of conditions. Thus we would expect to find large differences in the chemical properties and mechanical performances of different silks, even if these silks apparently perform similar tasks. Dragline, which doubles as a structural silk in the spider's web, is a good example to study this question by comparing the mechanical properties of silk produced by different spiders or in different conditions. After all, the mechanical properties do matter for web engineering and should be strongly selected. Moreover, stress-strain curves can be measured easily and with high accuracy.

### **2. Materials and Methods**

We studied the Major Ampullate silks of adult spiders of the following species: *Euprosthenops* sp (Pisauridae), *Cyrtophora citricola* (Araneidae), *Latrodectus mactans* (Theridiidae), *Araneus diadematus* (Araneidae) and *Nephila edulis* (Tetragnathidae). All spiders were raised and maintained under controlled conditions in our laboratory where they were kept in 50x30x30cm clear perspex boxes, except for *Nephila edulis* and *Araneus diadematus* which were kept in square frames (*N.e.* 50x50x10cm and *A.d.* 30x30x5cm). Every third day all spiders were fed flies (*Musca domestica*) and their webs were sprayed with tap water. The controlled environmental conditions of the rearing room were  $24.5 \pm 2^\circ\text{C}$  with  $50 \pm 5\%$  RH and L/D cycle 12/12hs; conditions in the measuring room were  $24 \pm 3^\circ\text{C}$  and  $25 \pm 3\%$  RH.

For our measurements of force-extension characteristics we used a custom-built micro scale materials testing instrument. The lower arm detected force (FORT 10 force transducer; World Precision Instruments) with a time resolution of 6 ms. The force transducer was calibrated with known weights for series of points throughout its working range and showed a linear force resolution on  $4 \mu\text{N}$ . The upper arm moved strictly vertically driven by Pen Motor Assembly (Hewlett Packard) with a resolution of  $7.5 \mu\text{m}$ . The instrument was controlled and recorded using LabVIEW (ver. 4.0 on Macintosh).

To reel silk, the spider was first anaesthetised with carbon dioxide and fixed with its ventral side upwards with a net and pins to an expanded polystyrene block. A hole in the net gave access to the spinnerets. To avoid any effects of anaesthesia, silk was not reeled until the animal was fully awake as judged by its struggles. The spider was placed under a dissection microscope and a single monofilament of major ampullate silk was reeled from one of the spigots onto a motor driven take-up spool. This spool was 8.5 cm

in diameter with 6 circumferential narrow rods giving thread sections of 3.0 cm. Two adjustable motors controlled the reeling speed and the advancing of the spool.

Samples of silk from the reeling spool were collected on a pair of dividers. The divider tips had been freshly coated with nail varnish and carefully moved with a micro manipulator to touch a silk thread on the spool. After a drying time of 5 minutes, a hot wire was used to cut the thread. This thread was then mounted on the materials testing instrument as follows. With a micro manipulator the thread (mounted on the dividers) was very carefully touched against the two hooks of the instrument. A fine droplet of Super Glue (cyanoacrylate) was used to glue the thread to the hooks. When the glue was completely dry the thread was cut outside the gauge length with a hot wire. Before testing, the thread was brought to zero tension position so that it neither sagged nor pulled the force transducer. The initial length of the thread fixed between the two hooks of the instrument was  $6.9 \pm 0.5$  mm. The silk thread was then stretched under standard conditions at a speed of  $3 \text{ mm min}^{-1}$  until it broke.

The data for extension and force were recorded at a sampling rate of 20 data points  $\text{sec}^{-1}$ . For each reeling of thread we measured (i) force-extension characteristics repeatedly (three to seven times on separate sections of an individual thread filament) and (ii) thread diameters on two further sections. For each stress-strain curve the breaking stress (stress), breaking elongation (elongation) and Young's modulus (modulus) at strain 0.02; were determined; with nominal (engineering) stress (GPa) being plotted against nominal strain. To measure thread diameter, we carefully - without in any way straining it - attached a length of silk thread on a pair of dividers to a SEM stub using nail varnish. The silk was sputtered with gold for 5 minutes and thread diameters were measured from SEM micrographs taken in our CamScan Maxim at 7kV; 10,000x. The measuring error was calculated to be about 5%. Silk diameters were measured six times on different locations on each thread section and an average was calculated for each thread.

The following measurements and experiments are presented in this paper:

- A comparison of dragline thread silks reeled at  $2.0 \text{ cm s}^{-1}$  from different species belonging to a diverse range of families: Pisauridae (*Euprosthenops* sp. ), Tetragnathidae (*Nephila edulis* ), Theridiidae (*Latrodectus mactans*) and Araneidae (*Cyrtophora citricola* and *Araneus diadematus* ) all reared from eggs in our laboratory under similar conditions. Note that *Euprosthenops*, *Latrodectus* and *Cyrtophora* build long-term (2-3 months), knock-down 3-D space webs that catch by breaking threads whereas *Araneus* and *Nephila* build short-term (1-5 days), sticky 2-D orb webs that catch by displacement and glue.
- A comparison of silk reeled at  $2.0 \text{ cm s}^{-1}$  from three different but related individuals within one species, i.e. *Nephila edulis*., reared in our laboratory under identical conditions.
- A comparison of silk produced by starved spiders and reeled at  $1.0 \text{ cm s}^{-1}$ . For this experiment we used three individuals of *N. edulis*. The experiment continued for 40 days and included two feeding periods (day 1 to 8 and day 28 to 40) and one starvation period (day 9 to 27). In the feeding periods the spiders were given 5 flies (*Musca domestica*) each day. Throughout the experimental period the spiders were given water every day. The weights of the spiders dropped during the starvation period and went up during the feeding periods. Silk was reeled from each of the spiders eight times: three times during the feeding periods and five times during the starvation period.
- The stress-strain characteristics of silk reeled at three different speeds from individuals of both *N. edulis* and *A. diadematus*. The spiders of each species were at comparable stages of their life cycle but of different sizes with average weights of  $389 \pm 58$  mg for *N. edulis* and weights of  $140 \pm 24$  mg for *A. diadematus*. Silk was reeled at speeds 0.4, 2.0 and  $8.0 \text{ cm s}^{-1}$  for *N. edulis* and 0.4, 2.0 and  $10.0 \text{ cm s}^{-1}$  for *A. diadematus*. To exclude carry-over effects, we randomised the order of testing.

Where applicable, we statistically analysed the results of experiments using a single-classification ANOVA (with JMP ver. 3.2.1 on Macintosh). For comparisons among pair of means in each data set we performed the Tukey-Kramer test at  $p=0.05$ .

### 3. Results

It is clear from our measurements that the different spider species produced draglines with rather different mechanical properties. Figure 1 shows stress-strain characteristics of silk reeled from *Euprosthenops* sp. (Pisauridae), *Latrodectus mactans* (Theridiidae), *Cyrtophora citricola* (Argiopidae), *Araneus diadematus* (Argiopidae) and *Nephila edulis* (Tetragnathidae). We found obvious and significant differences in stress-strain characteristics between the silks of the different species.

Our measurements further showed that the daily variability in dragline mechanical properties was rather large. Figure 2 shows the stress-strain characteristics of silk reeled from the same individual of *N. edulis* under identical conditions.

When we varied the conditions under which the spiders were kept, we found that this affected dragline mechanical properties in the measurements breaking stress (stress), breaking elongation (elongation) and Young's modulus (modulus) for threads from three individual of *N. edulis*. The starvation period was from day 9 to day 27. An ANOVA was performed for stress, elongation and modulus for each individual. This test showed that there were significant differences in each of the properties between the days of the experiment ( $p < 0.02$ ). Although stress and modulus varied from day to day, the variation did not seem to correlate in any way with the starvation period. However for individual A the comparisons of means showed that there was a significant difference in elongation between the days of the starvation period and the days of the feeding periods (Tukey-Kramer method at a significance level of 5%); elongation fell with starvation and increased again when the spiders had been fed. For individual B only day 20 and 22 in the starvation period differed significantly from the days in the feeding periods; elongation fell only towards the end of the starvation period. The elongation for individual C showed great variability which masked any potential correlation. We interpret our results to indicate that starvation can have an effect on break elongation of the silk, but apparently not on breaking stress and modulus.

When one variable of the reeling conditions was changed and we reeled at different speeds, we found a clear effect of reeling speed on the mechanical properties of this silk. We further found that the individual between-spider differences were not masked by this variable for breaking stress, breaking elongation and Young's modulus. Comparisons of means of those properties for silks reeled at the speeds of 0.4 and 8.0 cm s<sup>-1</sup> were performed for each individual. The result showed a consistent and a significant difference for all properties. Comparisons of means for 0.4 and 2.0 cm s<sup>-1</sup> showed only a significant difference in two cases (stress for individual B; elongation for individual A).

When we compared the mechanical properties of silks reeled at different speeds from both *N. edulis* and *A. diadematus*, we found that the silks of both species were similarly affected. In both cases, the faster reeling speed resulted in increased breaking stress and modulus but in decreased breaking elongation. However, underlying this effect was a more complex net of cross-correlations. Plotting not reeling speed against properties but, for the same data, plotting the intercorrelations of the different mechanical properties showed some very interesting differences between the two silks (Fig 4). All the regressions on figure 4 tested to be significant at a 5% level! The figure shows that, as stress increased (due to increased reeling speed) then the relative changes in modulus appeared to be identical for the two species. However, as elongation decreased (also due to increased reeling speed), then the relative changes of both, modulus and stress, appeared to differ between the two species: as elongation dropped, stress and modulus increased relatively faster for *A. diadematus* than for *N. edulis*. Thus even though the overall changes in stress, elongation and modulus appeared to be identical for the two species, our results show species-specific differences in the interactions between the different mechanical properties.

The stress-strain characteristics of silk reeled at our standard speed of 2.0 cm s<sup>-1</sup> from individual *N. edulis* and *A. diadematus* are shown in Figure 4. The ranges of breaking stress, breaking elongation and Young's modulus were, respectively, 1.06±0.10 GPa, 0.37±0.05 and 7.38±0.73 GPa (mean±S.D., n=30), for *N. edulis*. For *A. diadematus*, the averaged data for stress, elongation and modulus were, respectively, 1.08±0.16 GPa, 0.28±0.04 and 6.90±1.22 GPa (mean±S.D. n=30). Different individuals of *N. edulis* showed significant differences in stress and elongation ( $p < 0.0001$ ), but not in

modulus ( $p=0.33$ ). *A. diadematus* showed a significant difference between individuals for all properties ( $p<0.0001$ ). This result indicates that the mechanical properties of silk from different individuals within a species are not consistent but display some variation.

#### 4. Discussion

Firstly, we note the rather large variability in the mechanical properties of silk taken from the same individual under similar conditions. Work<sup>1</sup> made a similar observation but interpreted the variability he observed as inadequacy of his experimental procedures. We cannot apply the same explanation: our measuring equipment was highly accurate, our measuring conditions were fully controlled, as was our spider rearing and housing. Moreover, repeated control measurements on the same silk thread showed nearly no variation. Consequently we must explain the variability which we and Work observed by invoking variation of some non-standardised conditions or, more interesting, by assuming that each spider might produce silk with properties that it can and does change according to some requirements.

Secondly, our measurements of dragline silk from a range of spiders species showed that the mechanical properties between species can differ tremendously. It is obvious that both inter- and intra-species differences as well as inter- and intra-individual differences in mechanical properties are important variables. Consequently we must treat with caution interpretative comparisons between silks, even if they originate from different individuals of the same species.

One condition contributing to the observed inter-individual variability could be the state of nutrition of the spider. Starvation has been shown to have an effect on the web-geometry in orb-weaving spiders<sup>2</sup>; and it is likely that malnutrition will cause the amount of freely available aminoacids to decrease, which might affect silk chemistry<sup>3</sup> and, consequently, mechanical properties. Our results of the stress-strain properties of silk from starved spiders indicate that break elongation decreases with starvation, although our results were not entirely consistent for all individuals tested. The analysis is complicated by the unavoidable large time delay between control and experimental measurements. Thus any real starvation effects could have been masked by added variability originating from unknown and non-standardised factors.

Because of the absence of such confounding variables (i.e. long time spans between measurements), experiments with different reeling speeds provide a better experimental design than starvation to investigate specific conditions that also in nature might affect the mechanical properties of silks. Others have shown that reeling speed can have a measurable effect<sup>4-5</sup>. In our case, we reeled the threads (of known spiders) at highly controlled conditions with reeling speed as the only variable. The results from this experiment were highly consistent and thus reliable. Both, *A. diadematus* and *N. edulis* showed with increasing reeling speed (i) decreasing elongation coupled with (ii) increasing breaking stress and (iii) increasing modulus.

The explanation for this observation might lie in the production process of the silk: it is possible, indeed likely<sup>6,7</sup>, that with increased reeling speeds the molecules and liquid crystals of the silk precursor proteins become more and more orientated. And increased orientation might cause the silk to be stiffer and stronger but less extensible. We observed that *A. diadematus* seemed to be more sensitive to changes in reeling speed than *N. edulis*. This observation could be explained by the fact that adult *Nephila* females are much larger than *A. diadematus*, and consequently have a much larger silk production organ with bigger glands as well as wider spinning ducts and spigots. On the other hand, the observed differences in silk behaviour might lie in different packings of the silk molecules.

We have demonstrated big differences in the mechanical properties of Major Ampulate dragline silks between species taken from different families; this is not surprising since the spiders chosen have also different biologies. We have further demonstrated some differences between individuals of the same species; this, too, is to be expected since organisms typically show genetic variability in any trait that is not under extreme stabilising selection. Finally, we found variability in the same silk even within an individual. This shows a certain amount of flexibility on behalf of the spider and would indicate that the spider's silk production system is under constant supervision in order to perform rapid

adjustments. These can be induced by long-term effects (e.g. spider growth, not studied here), medium term effects (e.g. food intake, studied through starvation) or even short-term effects (e.g. drawing speed). We conclude that it is absolutely necessary to keep all conditions (and not only the conditions at measuring the silk) as constant as possible if one wants to compare the mechanical (and indeed chemical) properties of spider silks.

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## Figure captions

**Figure 1.** Stress-strain characteristics of silk reeled from spiders belonging to widely diverging taxa: *Euprosthenops* sp (Pisauridae) (1), *Cyrtophora citricola* (Araneidae) (2), *Latrodectus mactans* (Theridiidae) (3), *Araneus diadematus* (Araneidae) (4) and *Nephila edulis* (Tetragnathidae) (5). Each curve is a representative example taken from at least 25 tests of silk from different individuals within the species. Note the large inter-specific differences in drag- and structural line which might or might not correlate to web type : *Euprosthenops*, *Latrodectus* and *Cyrtophora* build 3-D space knock-down webs that catch by breaking threads and that have a long active life (several months) whereas *Araneus* and *Nephila* build 2-D orb webs that catch by sticking to the prey and swinging through the air and that have a short service life (a few days at most).

**Figure 2.** Stress-strain characteristics of silk from a single individual of *N. edulis*. The number by each curve denotes the day in a sequence. Note the large daily variability.

**Figure 3.** Comparison of the effect of three different reeling speeds on the mechanical properties of silk from *N. edulis* (filled symbols) and *A. diadematus* (empty symbols). The regressions are: Breaking stress-modulus (*A.d.*:  $y=0.313 + 8.451x$   $R^2=0.635$ ; *N.e.*:  $y=-0.230 + 7.436x$   $R^2=0.683$ ); Break elongation-modulus (*A.d.*:  $y=9.524 - 13.673x$   $R^2=0.675$ ; *N.e.*:  $y=10.642 - 8.093x$   $R^2=0.875$ ); Break elongation - Breaking stress (*A.d.*:  $y=0.938 - 1.040x$   $R^2=0.439$ ; *N.e.*:  $y=1.325 - 0.708x$   $R^2=0.541$ ).

**Figure 4.** Stress-strain characteristics of silk reeled from six individuals each of *N. edulis* (a) and *A. diadematus* (b). Each curve is a representative example taken from five tests. Note the large inter-individual differences.

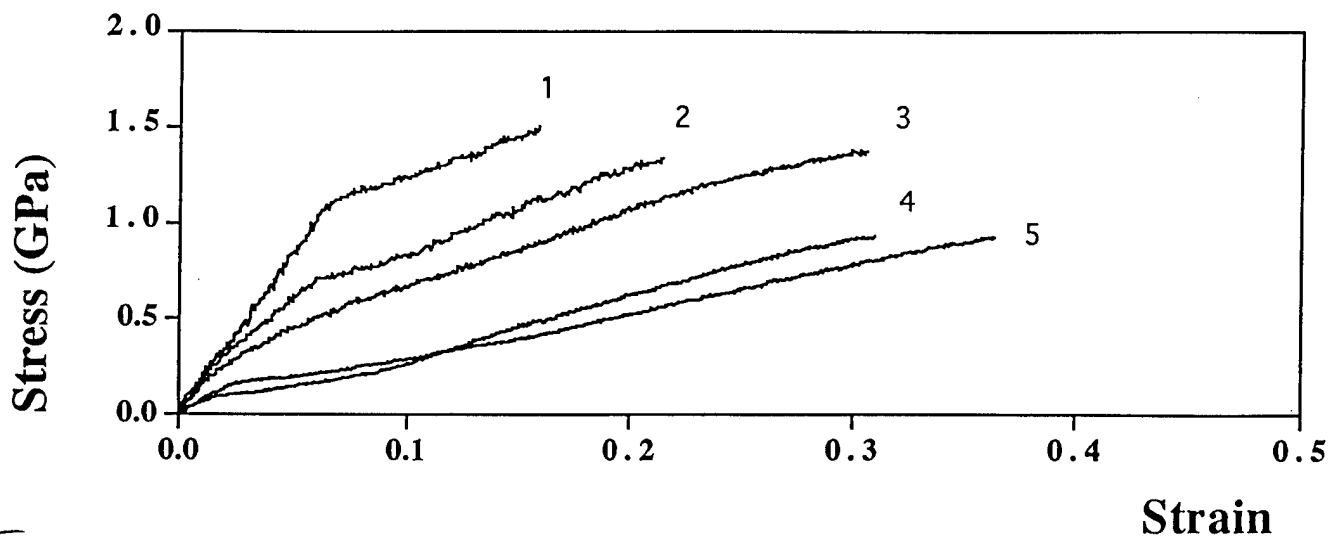


Fig 1

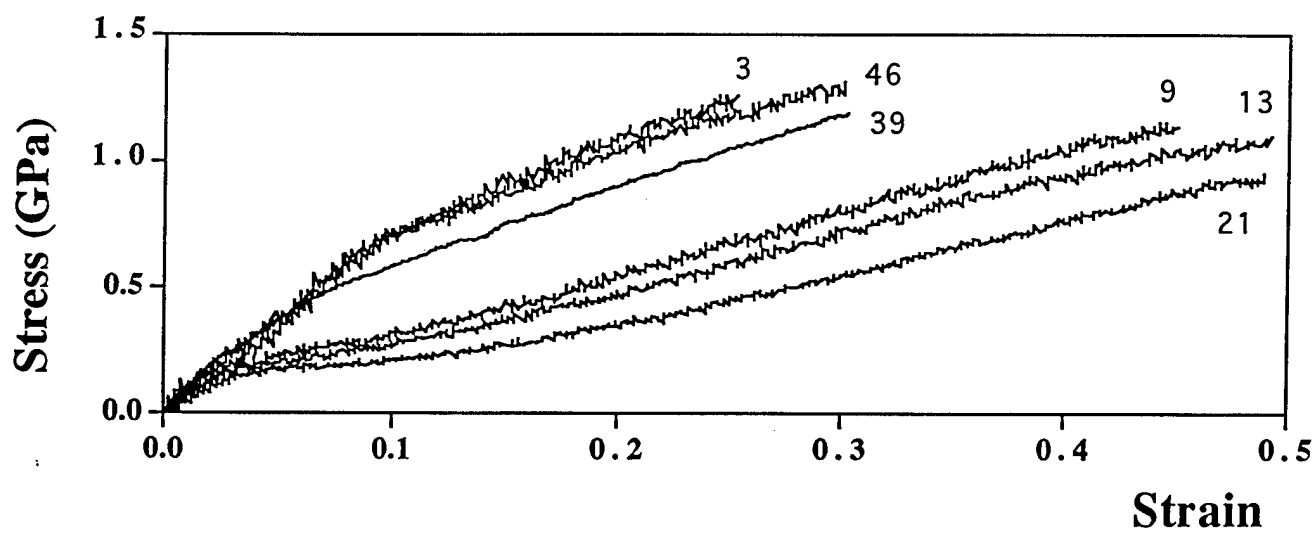


Fig 2



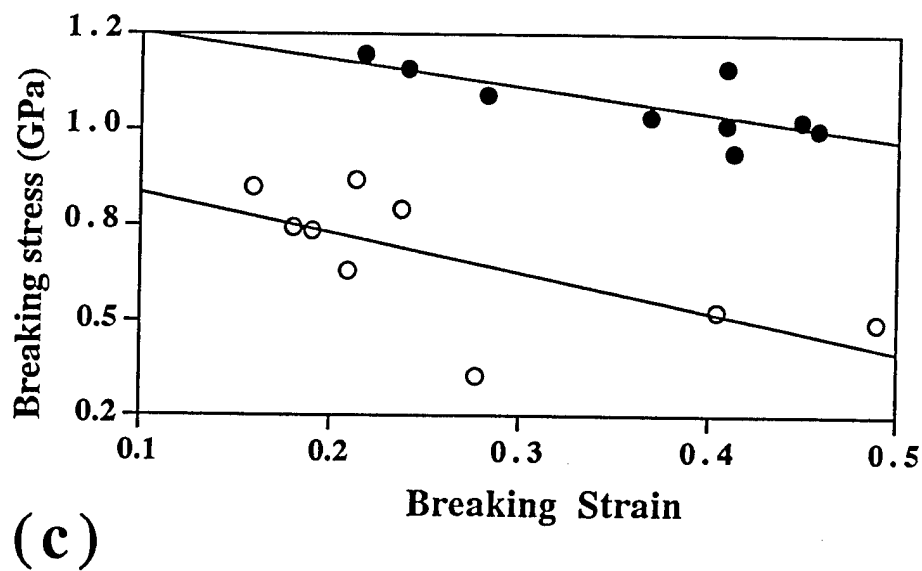
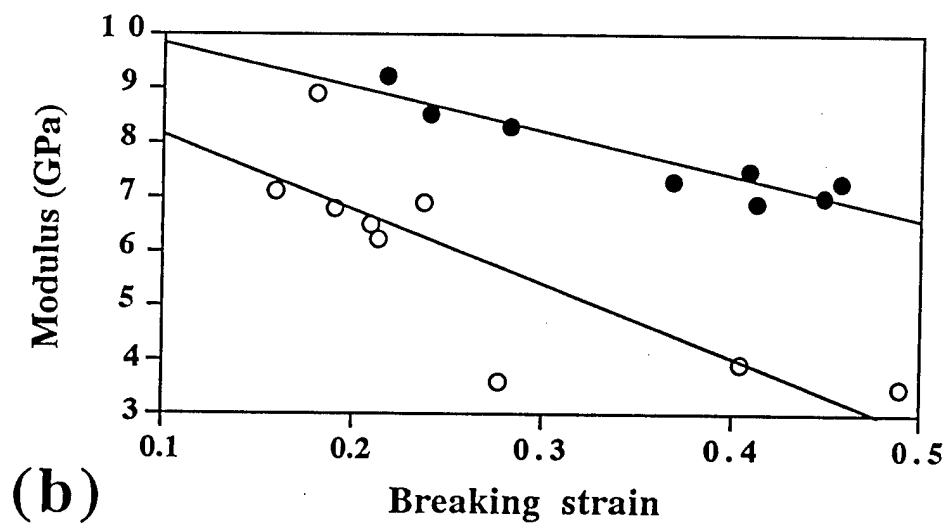
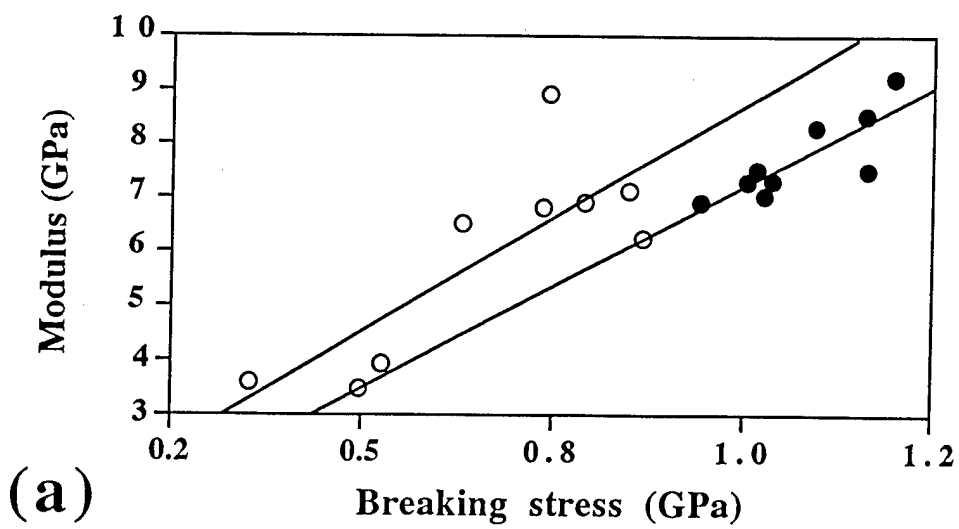
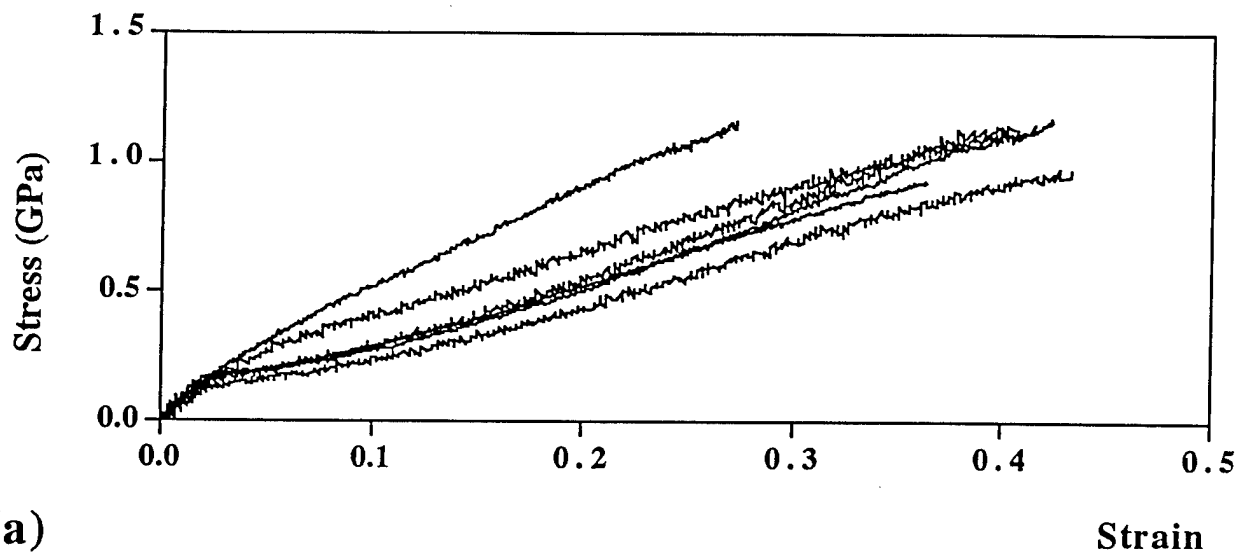
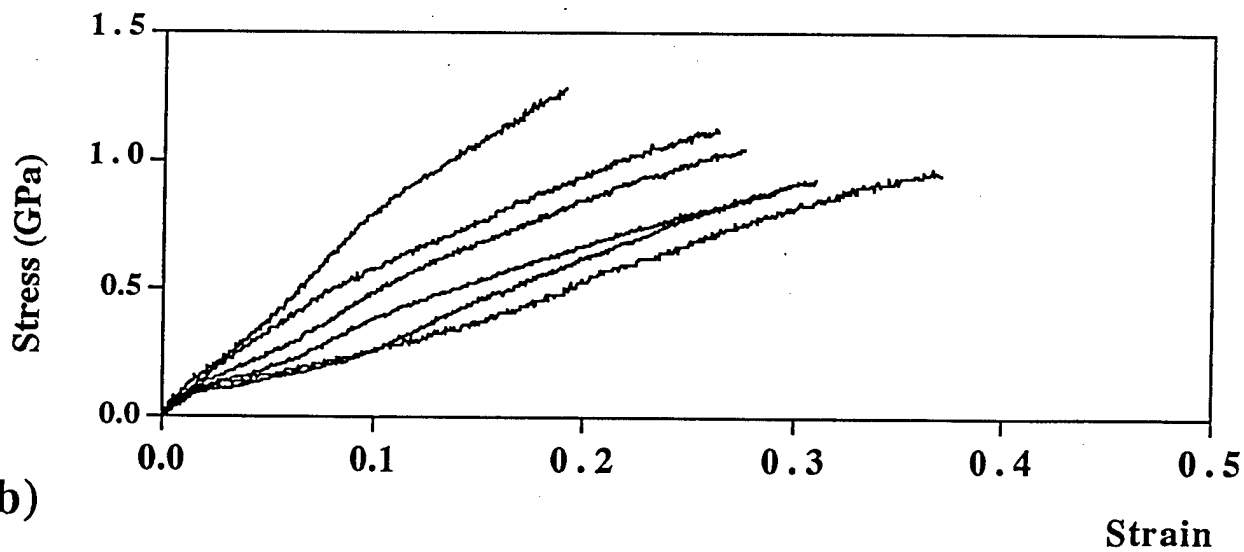


Fig 3



(a)



(b)

Fig 4